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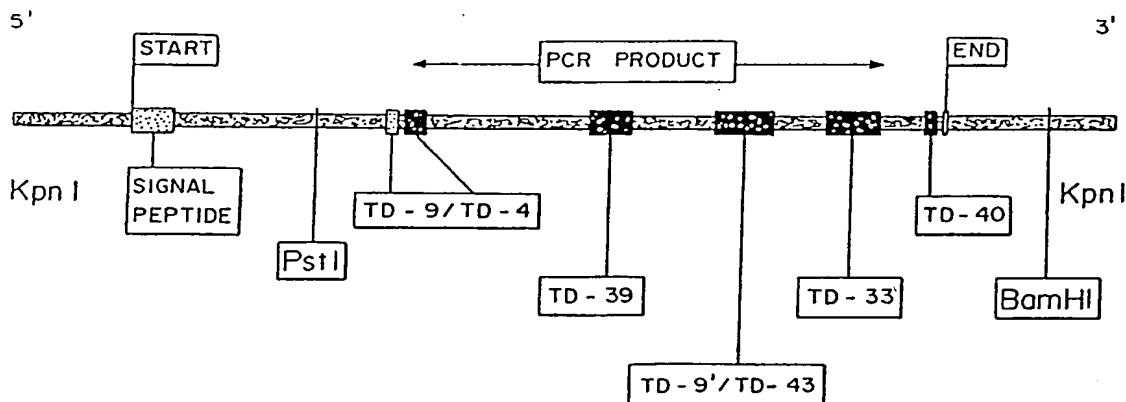
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(54) Title: HEPARINASE GENE FROM *FLAVOBACTERIUM HEPARINUM*

## (57) Abstract

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. The amino acid sequence reveals a 20-residue leader peptide. The gene was expressed in two expression systems in *E. Coli*.

HEPARINASE GENE FROM *FLAVOBACTERIUM HEPARINUM*

## Background of the Invention

This invention is generally in the area of heparinases and is specifically directed to the gene encoding heparinase I, expressed in *Flavobacterium heparinum*.

The United States government has rights in this invention by virtue of grant number 25810 from the National Institutes of Health.

Heparin is an anticoagulant that activates serine protease inhibitors (serpins), which play a key role in the blood clotting cascade, as described by Damus et al., *Nature* 246:355-357 (1973). According to Lindahl et al., *Trends Biochem. Sci.* 11:221-225 (1986), heparin is the most acidic natural polymer known to date. It consists of a major 1,4-linked disaccharide repeating unit of D-uronic acid 1,4- $\beta$ -D-glucosamine, and has an average of four negative charges (three sulfate groups and one carboxylate group) per monosaccharide unit. Heparin is both polydisperse, having an average molecular weight between 3,000 and 45,000 daltons, and heterogenous due to partial epimerization of D-glucuronic acid to L-iduronic acid and incomplete N- and O- sulfation, as reported by Kusche et al., *Proc. Natl. Acad. Sci.*, 77:6551-6555 (1980) and Comper, *Polymer Monograph* 7, 1981.

In addition, proteoglycans like heparin have a wide range of biological influences, including in blood chemistry, growth factor interaction and wound healing, interaction with basic structural proteins in the extracellular matrix and in cellular mediated immune responses. The basic nature of protein/peptide heparin/complex carbohydrate interaction is important. Although heparin seems fairly heterogenous, it is now quite clear that different heparin fractions exhibit distinct and unique properties indicating some

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activation energy of 4.5 kcal/mol, a  $k_m$  of  $8 \times 10^{-6}$  and a  $V_{max}$  of  $4 \times 10^{-7}$  M/min.

Heparin is often used in surgery to prevent blood clotting and to increase the compatibility of extracorporeal devices such as heart-lung and kidney dialysis machines. The enzymatic degradation of heparin by heparinase is sufficient to eliminate the anticoagulation properties of heparin in surgery. As described by Langer, et al. in *Biomaterials: Inter-facial Phenomenon and Applications, Adv. in Chem. Symposium Series*, Chap. 13, pp. 493-509 (1982), this property has led to the use of heparinase as an immobilized bioreactor in conjunction with heart-lung or kidney dialysis machines to deheparinize blood. Commercial application of the heparinase bioreactor is pending clinical trials.

A principal problem in the use of the heparinase bioreactor is the availability of sufficient amounts of pure heparinase to be immobilized onto a surface. This is primarily because the amount of heparinase constitutively expressed in *F. heparinum* is very low. Inducing expression of heparinase in *F. heparinum* with heparin is very expensive due to the amounts of heparin needed and the size of the fermentation to produce reasonable amounts of heparinase for any practical applications.

Cloning and expression of the heparinase gene is important in several ways. First, the only enzyme cloned and characterized to date which acts to depolymerise proteoglycans is heparinase. Second, heparin is the only anticoagulant commonly used in surgery so deheparinizing blood is an important medical problem. Moreover, heparinase catalyzed degradation of heparin into lower molecular weight heparin molecules can be used to yield products with specific anticoagulant activity, as discussed by

### Summary of the Invention

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. Two degenerate oligonucleotides, based on amino acid sequence derived from tryptic peptides of purified heparinase were used in the PCR with *Flavobacterium* genomic DNA as the template to generate a 600 base pairs probe. This probe was used to screen a pUC 18 *Flavobacterium* genomic library. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. Eleven different tryptic peptides (approximately 48% of the total amino acids) mapped into the ORF. The amino acid sequence reveals a 20-residue leader peptide.

Heparinase can be expressed from the gene. Additionally, the gene can be modified to produce heparinase with altered enzymatic activity, specificity, or binding properties. The sequence can also be used as a probe in the isolation of genes encoding other related enzymes.

### Brief Description of the Drawings

Figure 1 is a schematic representation of the PCR products Y1:C and D:C which are 600 and 160 basepairs, respectively. The 600 basepair PCR product was used as a template with D and C as primers to generate the 160 basepair D:C product.

Figure 2 is the restriction map of the genomic DNA pUC 18 plasmid, pRS.HEP51, having an insert containing the heparinase gene. The plasmid is 5631 bases long and has approximately 2300 bases of insert. The heparinase gene is in the *Kpn I*-*Kpn I* fragment.

Figure 3 is a *Kpn I*-*Kpn I* fragment map showing the heparinase gene structure with the different tryptic peptides mapping into the open reading frame. Six

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AAA ATG CCC TTT GCC CAG TTC CCT AAA GAT TGC TGG ATT  
ACT TTT GAT GTC GCC ATA GAC TGG ACG AAA TAT GGA AAA  
GAG GCC AAT ACA ATT TTG AAA CCC GGT AAG CTG GAT GTG  
ATG ATG ACT TAT ACC AAG AAT AAG AAA CCA CAA AAA GCG  
CAT ATC GTA AAC CAG CAG GAA ATC CTG ATC GGA CGT AAC  
GAT GAC GAT GGC TAT TAC TTC AAA TTT GGA ATT TAC AGG  
GTC GGT AAC AGC ACG GTC CCG GTT ACT TAT AAC CTG AGC  
GGG TAC AGC GAA ACT GCC AGA TAG (stop codon)

The following is the amino acid sequence (Sequence No.  
2) of heparinase:

Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln  
Leu Phe Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser  
Gly Asn Ile Pro Tyr Arg Val Asn Val Gln Ala Asp Ser  
Ala Lys Gln Lys Ala Ile Ile Asp Asn Lys Trp Val Ala  
Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp Asp  
Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu  
Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala  
Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr  
Ala Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val  
Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr His Tyr  
Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr  
Thr Phe Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn  
Ala Thr Thr Ile Phe Ala Gln Trp His Gly Ala Pro Ser  
Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile Lys Thr  
Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met  
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys  
Lys Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys  
Pro Asn Gly Trp Lys Val Glu Gln Gly Gly Tyr Pro Thr  
Leu Ala Phe Gly Phe Ser Lys Gly Tyr Phe Tyr Ile Lys  
Ala Asn Ser Asp Arg Gln Trp Leu Thr Asp Lys Ala Asp  
Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met Lys  
Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr  
Lys Met Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile  
Thr Phe Asp Val Ala Ile Asp Trp Thr Lys Tyr Gly Lys  
Glu Ala Asn Thr Ile Leu Lys Pro Gly Lys Leu Asp Val  
Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln Lys Ala

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for 24 hour. The reaction was terminated by heating the sample at 65°C for 2 minutes. The digest was separated by reverse phase HPLC using a gradient of 0 to 80% acetonitrile. The tryptic peptides were monitored at 210 and 277 nm.

The tryptic peaks were collected in Eppendorff tubes. Based on the homogeneity of the peptide peak, eight different peaks were sequenced using an Applied Biosystems sequencer, model 477, with an on-line model 120 PTH amino acid analyzer located in the Biopolymers lab, Center for Cancer Research, MIT. The sequences are set forth in Table I below. The designation (K,R) is used in Table I to indicate that trypsin cuts at either lysine or arginine residues. The asterisks in Table I represent amino acids that could not be determined. The peptide designated td Lx is the longest peptide sequenced having 38 residues. Native heparinase was also sequenced to determine the N-terminus amino acids.

Table I: Sequences of Tryptic Peptides of Heparinase

<u>Peptide</u>	<u>Amino Acid Sequence</u>
td 04	(K, R) G I C E Q G S S R
td 09	(K, R) T V Y H Y G K
td 09'	(K, R) T S T I A Y K
td 21	(K, R) F G I Y R
td 33	(K, R) A D I V N Q Q E I L I G R D D * G Y Y F K
td 39	(K, R) I T Y V A G K P N G N K V E Q G G Y P T L A F *
td 43	(K, R) M P F A Q F P K D C W I T F D V A I D * T K
td 40	(K, R) N L S G Y S E T A R
tdm4	K N I A H D K V E K K
td 72	K T L S I E E F L A L Y D R
td Lx	R S Y T F S V Y I P S S F P D N A T T I F A W H G A P S R T L V T P E I K

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primers:

- A     5'- ATI AA(T/C) CA(A/G) GA(A/G)ATI (C/T)TI  
         AT(T/C/A) GG -3'
- B     5'- CCIATIA(G/A) IAT (T/C)TC (T/C)TG (T/C)TG  
         (A/G)TT ICA (A/C)AT
- C     5'- CCIATIA(G/A) IAT (T/C)TC (T/CTG (T/C)TG  
         (A/G)TT ICA (T/G)AT -31

Of the six RHPLC peaks initially sequenced (Table I), three were chosen for primer design. Three sets of primers were designed (Table II). The PCR product of the combination the primers td43 and td33 was about 150 base pairs in length. The combination of td4 and td33 primers were about 600 base pairs. Primer td43 was 5' to primer td33 and primer td4 was 5' to td43 primer. Using the PCR product of td4 and td33 as a template and td43 and td4 as primers the predicted 150 base pair product was obtained confirming that td43 was between td4 and td33.

The 600 basepair product shown in Figure 1 represents about 51% of the approximated total 1170 base pairs for the heparinase gene, assuming 43,000 dalton for heparinase and a 110 dalton average amino acid with a molecular weight corresponding to about 390 amino acids times three which is 1170 bases.

The 600 base pair probe was chosen for screening a pUC 18 library by high stringency colony hybridization. Two positive clones were identified which were carried through for three rounds of colony purification.

#### Genomic DNA, RNA, and Plasmid Library

The *F. heparinum* genomic DNA was isolated by the A.S.A.P.™ kit (Boehringer Mannheim, Indianapolis, IN) with the following modifications. The DNA was desalted over a Sephadex™ G-50 column (Nick column, Pharmacia, Piscataway, NJ) and concentrated using a Centricon™ P-30 (Amicon Division, Beverly, MA) to a final volume of 100 l. From  $1 \times 10^9$  cells, 105-115 g of DNA typically were obtained. Total cellular mRNA was isolated using the

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the PCR product run was isolated from a low melt agarose gel, denatured by boiling at 95°C for 10 minutes, and then chilled on ice. To the denatured DNA were added 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), random hexanucleotides in the reaction buffer, and 50 µCi of <sup>32</sup>PdCTP(3000 Ci/mmol). The reaction was carried with Klenow for 30 minutes at 37°C and terminated using 0.2 M EDTA. Following the labelling reaction, the labelled probe was purified from the free nucleotide by using a Sephadex G-50 column (Nick Column, Pharmacia, Piscataway, NJ). The colonies were screened with the labelled probe using standard colony hybridization procedures as described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, incorporated herein by reference.

Two positive clones were isolated and the plasmids tested for their ability to generate the 600 basepair PCR product. Both of the clones tested positive and were further characterized by restriction mapping. Clone pRS Hep 51 is a 2.3 kb insert in pUC 18 (shown in Figure 2) with a *Kpn*-*Kpn* fragment of about 1.6 kb. This fragment was a positive template for generating a 600 basepair PCR product. The *Kpn*I-*Kpn*I fragment of pRS 51 was subcloned into M13 and sequenced.

#### DNA Sequencing

DNA sequencing was performed using phage M13 and employing the dideoxyadenosine 5'-alpha-<sup>35</sup>S-triphosphate and Sequenase (US Biochemical Corp, Cleveland, OH) as described by the manufacturer. The sequence data was obtained using successive nested deletions in M13 using T4 DNA polymerase as per Cyclone I Biosystems (International Biotechnologies Inc., New Haven, CT) or sequenced using synthetic oligonucleotide primers.

The sequence reveals a single, continuous open reading frame (ORF) of 1152 basepairs corresponding to



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the Omp A pIN vector with the *E. coli* periplasmic leader sequence. DH5 $\alpha$  was transformed and expression was induced with 1 mM IPTG for 3-5 hours.

As shown in Table III, the construct of the Omp A expression system results in two extra amino acids at the amino terminal of the heparinase gene, Gly and Ile. The heparinase sequence begins with a Gln.

#### The pKK expression system

The pKK expression system is used for over-expression of proteins in accordance with the methods of Brosius and Holy, *Proc. Natl. Acad. Sci.*, 81: 6929 (1984) and Jaffe et al. , *Biochem.* 27:1869 (1988), incorporated by reference herein. This system contains a strong tac promotor which, in appropriate hosts, is regulated by the lac repressor and induced by the addition of IPTG, as in the Omp A system. The plasmid pKK223-3 has a pUC 8 multiple cloning site and a strong rrnB ribosomal terminator immediately following the tac promotor. The ribosomal binding site of the plasmid was utilized by cloning the heparinase gene into a *Sma*I site, which is about 12 bases from the start codon ATG. Like the Omp A construction, the heparinase insert is obtained by PCR with *Sma*I and *Hind*III restriction sites at the N and the C terminals of the protein. As shown in Table III, the native heparinase leader sequence was used for over-production into the periplasm.

Periplasmic proteins of *E. coli* were isolated by osmotic shock. Briefly, 1.5 ml of cells were centrifuged after induction and washed with 10 mM Tris pH 7.5. The cells were then suspended in 20% sucrose in 10 mM Tris pH 7.5 and 5  $\mu$ l of 0.5 M EDTA. After a five minute incubation on ice, the cells were centrifuged and osmotically shocked by adding approximately 150  $\mu$ l water. The periplasmic extract was used to determine enzyme activity. Heparinase activity was determined by monitoring the wavelength at 232 nm and by the Azure A

Table III: Design of OmpA and pKK plasmids for expression of Recombinant Heparinase in *E.coli*

Omp A secretion Expression system														
N	Gly	Ile	Gln	Lys	Thr	Ala	Arg	End	C					
XXX	GGA	ATT	CAG	AAA	-----	ACT	GCC	AGA	TAG	GGATCCXXX				
XXX	CCT	TAA	GTC	TTT	-----	TGA	CTT	ACT	ATC	CCTAGGXXX				
EcoRI					Bam HI									
pKK over-Expression system														
N	Met	Lys	Lys		Ala	Arg	End							
XXX	Taa	CCC	GGG	ATG	AAA	AAA	-----	GCC	AGA	TAG	AAG	CTT	CCG	XXX
XXX	ATT	GGC	CCC	TAC	TTT	TTT	-----	CGG	TCT	ATC	TTC	GAA	GGC	XXX
					Sma I					Hind III				

of the peak profiles and some peaks which were isolated and sequenced.

A positive signal was obtained for the isolated *F. heparinum* mRNA using the 600 basepair probe derived from the PCR which has been used for isolating the heparinase gene, confirming that the gene isolated was a *F. heparinum* gene cloned in *E. coli*.

The expressed heparinase appeared to have at least some heparinase activity.

The sequence can be modified to alter specific enzymatic activity or binding specificity or affinity by substitution of one or more amino acids, using site directed mutagenesis or substitution of oligomers into the sequence encoding the heparinase. Methods and materials to accomplish this are known to those skilled in the art. The modified gene is then expressed and the product routinely screened for the altered activity.

Although described with reference to two specific expression systems, other expression systems are well known and commercially available. The heparinase gene can be expressed in these systems, using similar vectors and signal peptides or leader sequences.

Modifications and variations of the present invention will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the following claims.

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## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 404-815-6508  
(B) TELEFAX: 404-815-6555

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1379 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Flavobacterium heparinum

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTTTTGGGA	GCAAAGGCAG	AACCATCTCC	GAACAAAGGC	AGAACCAGCC	TGTAAACAGA	60
CAGCAATTCA	TCCGCTTTCA	ACCAAAGTGA	AAGCATTTAA	TACAATACCA	GAATGTCCGA	120
TTTCCCTTTC	AGCGTACTTT	TTGGGTAAAT	AACCAATAAA	AACTAAAGAC	GGATGAAAAA	180
ACAAATTCTA	TATCTGATTG	TACTTCAGCA	ACTGTTCTCT	TGTTGGGCTT	ACGCCCAGCA	240
AAAAAAATCC	GGTAACATCC	CTTACCGGGT	AAATGTGCAG	GCCGACAGTG	CTAAGCAGAA	300
GGCGATTATT	GACAACAAAT	GGGTGGCAGT	AGGCATCAAT	AAACCTTTATG	CATTACAATA	360

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Flavobacterium heparinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe Leu
 1      5      10      15

Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro Tyr Arg
20      25      30

Val Asn Val Gln Ala Asp Ser Ala Lys Lys Gln Lys Ala Ile Ile Asp Asn
35      40      45

Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp
50      55      60

Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu Leu Lys
65      70      75      80

Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala Gly Glu Thr Lys Gly
85      90      95

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Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr Phe Asp Val Ala  
 290 295 300  
 Ile Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn Thr Ile Leu Lys Pro  
 305 310 315 320  
 Gly Lys Leu Asp Val Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln  
 325 330 335  
 Lys Ala His Ile Val Asn Gln Gln Glu Ile Leu Ile Gly Arg Asn Asp  
 340 345 350  
 Asp Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser  
 355 360 365  
 Thr Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg  
 370 375 380

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Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro		
20	25	30
Tyr Arg Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile		
35	40	45
Ile Asp Asn Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala		
50	55	60
Leu Gln Tyr Asp Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr		
65	70	75
Arg Phe Glu Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala		
80	85	90
Ala Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr Ala		
95	100	105
Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val Tyr Gln Asn		
110	115	120
Ala Gln Lys Leu Lys Thr Val Tyr His Tyr Gly Lys Gly Ile Cys		
125	130	135
Glu Gln Gly Ser Ser Arg Ser Tyr Thr Phe Ser Val Tyr Ile Pro		
140	145	150
Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile Phe Ala Gln Trp His		
155	160	165
Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile		
170	175	180
Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met		
185	190	195
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys Lys Asp		
200	205	210
Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly Trp		
215	220	225
Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser		
230	235	240
Lys Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu		
245	250	255
Thr Asp Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu		
260	265	270
Val Met Lys Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala		
275	280	285

9. The nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a heparinase having a specific activity different from the specific activity of the heparinase encoded by Sequence No. 1.

10. The nucleic acid molecule of claim 1 in a procaryotic cell other than *F. heparinum* which is capable of expressing the molecule.

11. The nucleic acid molecule of claim 11 in a procaryotic cell cultured under low sulfate conditions which is capable of expressing the molecule.



FIG. 1

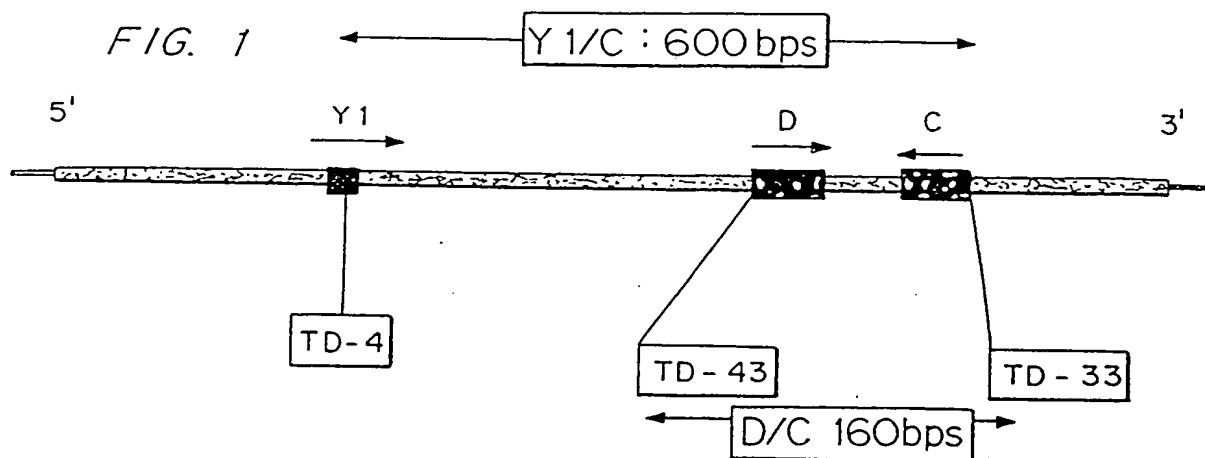


FIG. 2

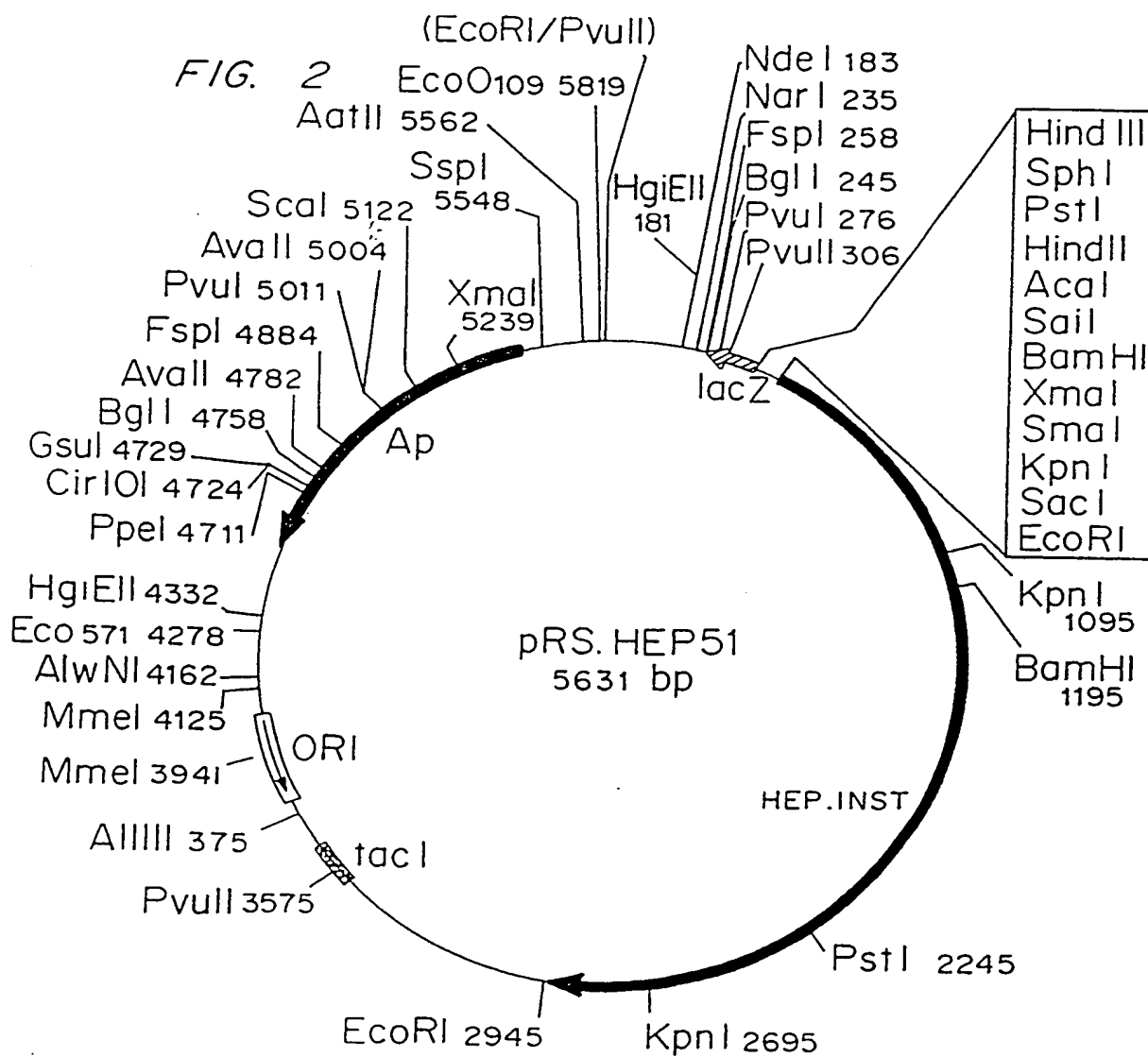
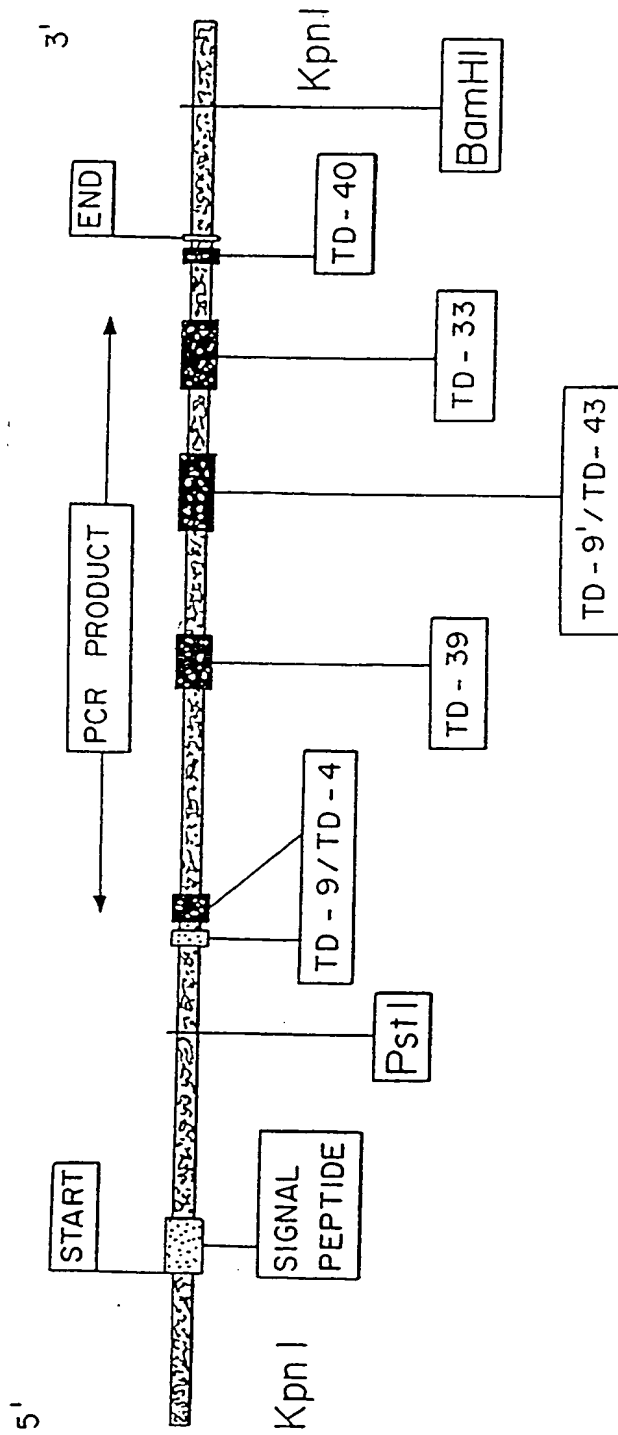


FIG. 3



## INTERNATIONAL SEARCH REPORT

PCT/US 92/09124

International Application 1

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/60		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,8 912 692 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 28 December 1989 see page 18, line 30 - page 20, line 9 ---	1-11
X	Week 9125, Derwent Publications Ltd., London, GB; AN 91-180925 & JP,A,3 108 486 (SHINGIJUTSU KAIHATSU) 8 May 1991 see abstract -----	1-11
<sup>10</sup> Special categories of cited documents: <ul style="list-style-type: none"> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the international filing date</li> <li>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> <li>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</li> <li>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</li> <li>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</li> <li>"&amp;" document member of the same patent family</li> </ul>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 19 FEBRUARY 1993		Date of Mailing of this International Search Report 09.03.93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer CUPIDO M.